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PGC1β Activates an Antiangiogenic Program to Repress Neoangiogenesis in Muscle Ischemia

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SUMMARY

Revascularization of ischemic skeletal muscle is governed by a balance between pro- and antiangiogenic factors in multiple cell types but particularly in myocytes and endothelial cells. Whereas the regulators of proangiogenic factors are well defined (e.g., hypoxia-inducible factor [HIF]), the transcriptional pathways encoding antiangiogenic factors remain unknown. We report that the transcriptional cofactor PGC1β drives an antiangiogenic gene program in muscle and endothelial cells. PGC1β transcriptionally represses proangiogenic genes (e.g., *Vegfc*, *Vegfd*, *Pdgfb*, *Angpt1*, *Angpt2*, *Fgf1*, and *Fgf2*) and induces antiangiogenic genes (e.g., *Thbs1*, *Thbs2*, *Angstat*, *Pedf*, and *Vash1*). Consequently, muscle-specific PGC1β overexpression impairs muscle revascularization in ischemia and PGC1β deletion enhances it. PGC1β overexpression or deletion in endothelial cells also blocks or stimulates angiogenesis, respectively. PGC1β stimulates the antiangiogenic genes partly by coactivating COUP-TFI. Furthermore, proangiogenic stimuli such as hypoxia, hypoxia-mimetic agents, and ischemia decrease PGC1β expression in a HIF-dependent manner. PGC1β is an antiangiogenic transcriptional switch that could be targeted for therapeutic angiogenesis.

INTRODUCTION

Skeletal muscle ischemia is a common problem in diseases such as diabetes, obesity, atherosclerosis, and aging, leading to peripheral vascular disease or critical limb ischemia (Baumgartner et al., 2005; Chi et al., 2011; Varu et al., 2010). Although inducing "therapeutic neoangiogenesis" to oppose ischemic muscle damage is a desirable treatment strategy, targeting individual angiogenic factors does not effectively revascularize infarcted muscles (Cao et al., 2005; Lekas et al., 2006; Simons and Ware, 2003; van Weel et al., 2008). Indeed, angiogenesis and vascularization constitute a complex process involving pro- and antiangiogenic factors that must be regulated in coordination, which makes it difficult to mimic the process with individual angiokines (Carmeliet and Jain, 2011). Proangiogenic factors such as vascular endothelial growth factor alpha (Vegfa) promote angiogenesis by triggering endothelial cell proliferation, migration, and differentiation (Carmeliet and Jain, 2011). On the other hand, antiangiogenic factors such as thrombospondin 1 (Thbs1) and Thbs2 limit angiogenesis by opposing Vegfa and endothelial cell proliferation and migration, as well as by inducing endothelial cell apoptosis (Lawler and Lawler, 2012). Uncovering the regulatory pathways that determine the balance between the pro- and antiangiogenic factors in muscle will yield candidates for therapeutic neoangiogenesis.

Various proangiogenic transcriptional pathways are defined in the skeletal muscle. A classic example is the master angiogenic regulator hypoxia-inducible factor (HIF) (Semenza, 2012), which when activated via adenoviral gene transfer or with synthetic agents induces neoangiogenesis in ischemic muscle (Milkiewicz et al., 2004; Pajusola et al., 2005; Sarkar et al., 2009). Recently, nuclear receptors and their coactivators have emerged as proangiogenic regulators in skeletal muscle. For example, the nuclear receptor cofactor peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α) promotes muscle angiogenesis, possibly via estrogen-related receptor alpha (ERRα) or HIF (Arany et al., 2008; O'Hagan et al., 2009). Similarly, nuclear receptors, including ERRγ, peroxisome proliferator-activated receptor alpha (PPARα), and PPARδ, also stimulate ischemic neoangiogenesis by inducing stimulatory angiogenic factors such as Vegfa (Gaudel et al., 2008; Han et al., 2013; Matsakas et al., 2012b; Salehi et al., 2012). However, skeletal muscles also express a battery of inhibitory angiogenic factors that define the muscle vasculature, such as Thbs1, Thbs2, angiostatin (Angstat), vasohibin 1 (Vash1), and pigment epithelium-derived factor (PEDF) (Kivelä et al., 2006; Olfert and Birot, 2011). Surprisingly, how these angiostatic factors are transcriptionally regulated and affect ischemic muscle neoangiogenesis remains unclear.

In this study, we investigated the role of the transcriptional coactivator PGC1β in muscle neoangiogenesis. We report that PGC1β drives an antiangiogenic gene program in muscle and endothelial cells that involves repression of proangiogenic

factors and induction of antiangiogenic factors, which limits ischemic skeletal muscle revascularization. Our findings have implications for therapeutic angiogenesis in skeletal muscle. They may also be relevant for cardiac myopathies, retinopathy, and cancer, where neoangiogenesis is deregulated.

RESULTS

Regulation of the Antiangiogenic Gene Program by PGC1β in Muscle Cells

To obtain insight into the regulation of angiogenic factors by PGC1β, we made stable C2C12 cells overexpressing PGC1β or control vector. The PGC1β-overexpressing cells had higher levels of PGC1β mRNA (8- to 10-fold; Figure 1A, upper panel) and protein (Figure 1A, lower panel) compared with the control cells. A known gene target of PGC1β, cytochrome c (*Cycs*), was induced in PGC1β-overexpressing muscle cells, demonstrating the functionality of the overexpressed cofactor (Figure S1A). Another study reported that PGC1β induces Vegfa in skeletal muscles (Rowe et al., 2011). Accordingly, we found that Vegfa gene and protein expression was increased in C2C12 cells overexpressing PGC1β (Figures S1B and S1C). Although Vegfa is an important proangiogenic factor, there are several other pro- and antiangiogenic factors that collectively orchestrate the muscle angiogenic response (Kivelä et al., 2006; Olfert and Birot, 2011). Therefore, we subjected the control and PGC1β-overexpressing cells to an angiogenesis gene-expression analysis using a PCR-based gene array. The array profiles the expression of 84 genes known to regulate angiogenesis. The angiogenic profiling revealed that proangiogenic genes were predominantly repressed by PGC1β. At the same time, a battery of antiangiogenic genes were induced by PGC1β (Figure 1B; Table S1). To further elaborate these findings, we performed quantitative PCR (qPCR) for individual gene sets of the proangiogenic genes fibroblast growth factor 1 (*Fgf1*), *Fgf2*, platelet-derived growth factor beta (*Pdgfb*), angiopoietin 1 (*Angpt1*), *Angpt2*, *Vegfc*, and *Vegfd*, as well as the antiangiogenic genes *Angstat*, *Thbs1*, *Thbs2*, collagen 18a1 (the proteolytic fragment of which is antiangiokine Endostatin), *Vash1*, and *Pedf*. We found that PGC1β repressed proangiogenic genes (Figure 1C) and induced antiangiogenic genes (Figure 1D). These results demonstrate that PGC1β triggers a net antiangiogenic program in the muscle cells that results from the induction of antiangiogenic genes and repression of proangiogenic genes.

PGC1β Negatively Regulates Neoangiogenesis in Muscle Ischemia

The above results obtained with cultured cells indicate that PGC1β might signal muscle to preferentially secrete antiangiogenic factors to limit angiogenesis and muscle vascularization. To physiologically test the effect of activating PGC1β in muscle cells on the muscle vasculature, we generated transgenic (TG) mice selectively overexpressing PGC1β in skeletal muscles using the human alpha skeletal actin promoter (Muscat and Kedes, 1987). The structure of the transgene and the cloning strategy for transgene generation are described in Figures S2A and S2B. Briefly, the whole transgene was excised from the cloning vector by restriction digestion (Figure S2C), purified, and microinjected into oocytes to obtain mouse lines positive for the transgene (Figure S2D). From several TG lines obtained, the one that exhibited the lowest overexpression of PGC1β in the skeletal muscle was selected for further examination. The overexpression of PGC1β in the skeletal muscles at the gene/protein level was confirmed in the tibialis anterior (TA), soleus, quadriceps, and gastrocnemius (Figures S3A and S3B). It was previously reported that PGC1β induces genes linked to myofiber type (myosin heavy chain type IIx [MHC-IIx]), mitochondrial respiration (cytochrome c oxidase subunit Vb [*Cox5b*], ATP synthase, H^+ transporting, mitochondrial F1 complex, O subunit [*Atp5o*], *Cycs*, NADH dehydrogenase [ubiquinone] 1 alpha subcomplex 5 [*Ndufa5*]) and fatty acid oxidation (medium-chain acyl-CoA dehydrogenase [*Acadm*], carnitine palmitoyltransferase 1 [*Cpt1*], *Cpt2*, and cluster of differentiation 36 [*Cd36*]), and induces mitochondrial biogenesis in skeletal muscle (Arany et al., 2007). Similarly, in our muscle-specific PGC1β TG mice, we found that the aforementioned PGC1β target genes were induced in the TG muscles at both the transcript (Figures S3C–S3E) and protein (Figure S3F) levels, confirming an enhanced PGC1β signaling in the skeletal muscle of the TG mice. Additionally, PGC1β overexpression resulted in increased mitochondrial biogenesis and oxidative myofibers (Figures S3G and S3H), imparting red coloration to the TG mice (Figure S3I), as previously reported (Arany et al., 2007). Furthermore, we eliminated the possibility that PGC1β overexpression in the skeletal muscle might lead to muscle damage due to transgene overexpression by demonstrating healthy morphology and comparable serum creatine kinase levels (Figures S3J and S3K).

In these PGC1β TG mice and wild-type (WT) littermates, we next examined the expression of candidate pro- and antiangiogenic genes in the TA. We found that the expression of several proangiogenic genes (*Fgf2* and the *Vegfa* isoforms *Vegfa121*, *Vegfa165*, and *Vegfa189*) was predominantly downregulated in the TG compared with the WT TA muscles (Figure 2A). Vegfc and Ang2 did not change significantly. Note that the repression of proangiokines seemed to be more complete in adult murine muscle cells than in the cultured muscle cells, as even expression of the Vegfa isoforms was repressed in the former (Figures 2A and S1). On the other hand, the expression of antiangiogenic genes (*Thbs1*, *Thbs2*, *Pedf*, *Angstat*, *Vash1*, and Endostatin) was induced in the TG TA (Figure 2A). In correlation with gene expression, PGC1β repressed or induced pro- (FGF2) and antiangiogenic (THBS1, THBS2, ENDOSTATIN, and PEDF) factors, respectively, at the protein level (Figure 2B). Therefore, muscle PGC1β transcribes a net antiangiogenic signal that might in a paracrine fashion limit muscle neoangiogenesis in mice.

We tested this idea by first performing an angiogenesis tube-formation assay with TA homogenates obtained from WT and muscle-specific PGC1β TG mice. In this assay, tube formation in human umbilical vein endothelial cells (HUVECs) is used as a measure of angiogenesis. We found that whereas the control HUVECs treated with WT TA homogenates were able to differentiate into vessel-like structures, this effect was abolished in HUVECs treated with TG TA homogenates (Figure S4A). These findings confirm that PGC1β transcriptionally encodes antiangiogenic signaling in the skeletal muscle that can inhibit angiogenesis.

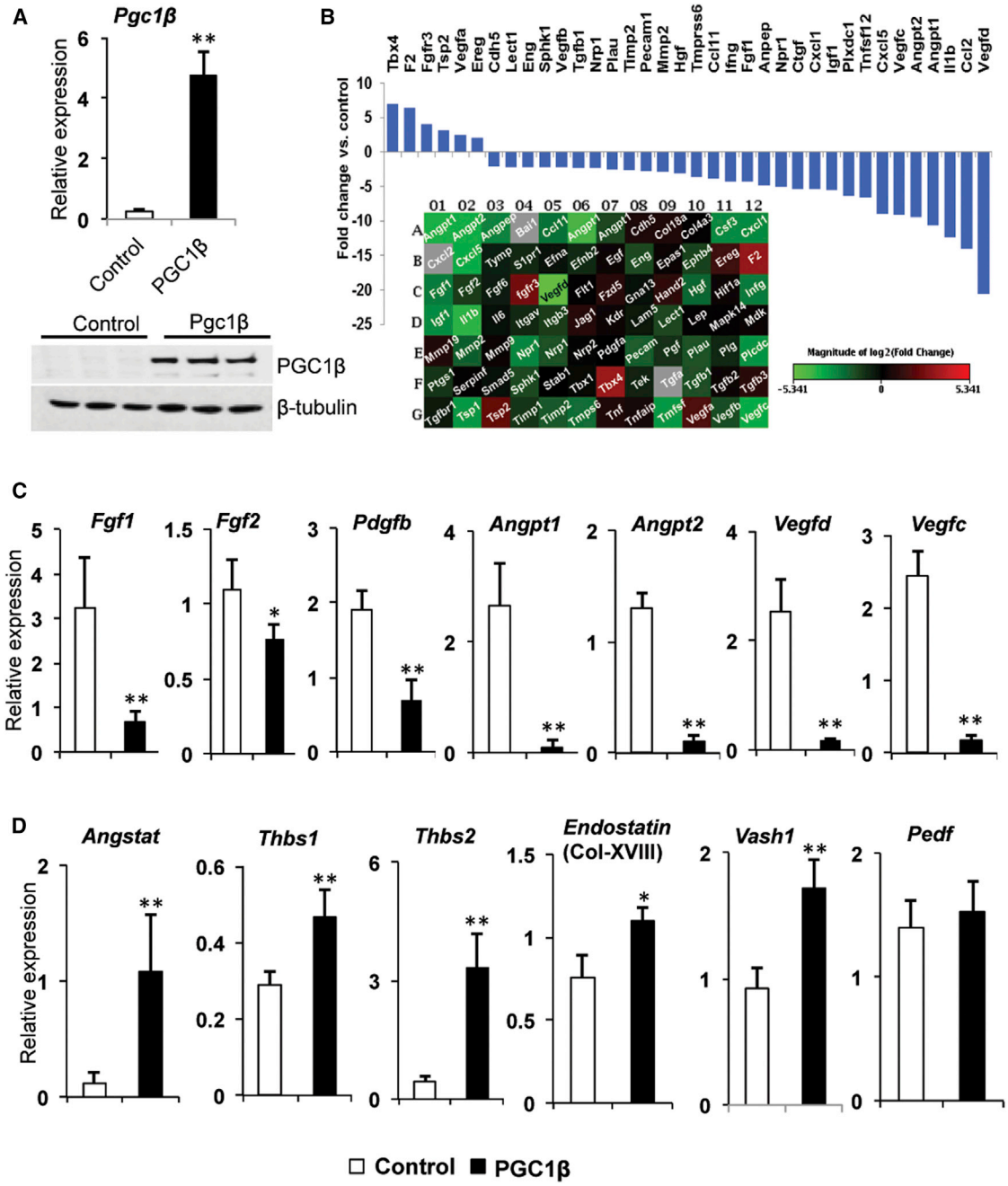

Figure 1. Regulation of Angiogenic Gene Expression by PGC1β

(A) PGC1β mRNA (top) and protein (bottom) expression in empty vector (control) and PGC1β-overexpressing C2C12 myotubes.

(B) Angiogenic PCR array data shown as fold change (≥2-fold and $p < 0.05$) for angiogenic gene expression in PGC1β-overexpressing C2C12 myotubes compared with control cells. Heatmap-based expression of all genes studied in the array, showing either repression (green) or induction (red).

(C and D) Relative mRNA expression of proangiogenic (C) and antiangiogenic (D) genes (n = 6, mean ± SD). *$p < 0.001$ and **$p < 0.000001$ (unpaired Student's t test).

See also Figure S1 and Table S1.

Because the aforementioned data pointed to an antiangiogenic role for PGC1β, we asked whether and how PGC1β might regulate ischemic muscle neoangiogenesis. We focused on a preclinical model for peripheral vascular disease, in which unilateral hindlimb ischemia is surgically induced in mice by ligating the femoral vessels in the left hindlimb (Matsakas et al., 2012b). The right hindlimb serves as a nonischemic control. The ischemic hindlimb undergoes spontaneous

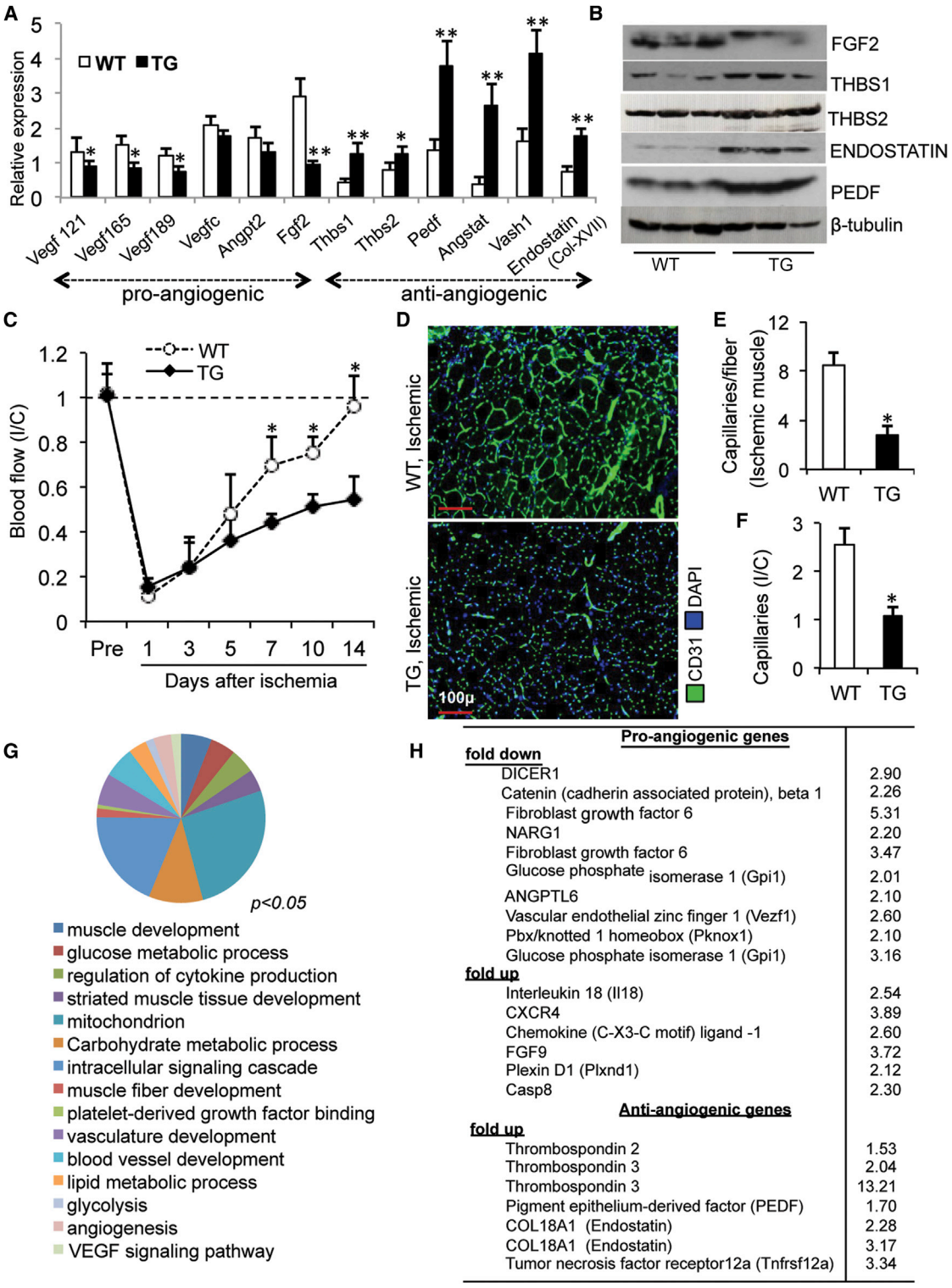

Figure 2. PGC1β Negatively Regulates Ischemic Revascularization

(A) Relative mRNA expression of pro- and antiangiogenic genes in TA from TG and WT mice (n = 5, mean ± SD). *p < 0.05 and **p < 0.001 (unpaired Student's t test).

(B) Representative western blots of FGF2, THBS1, THBS2, ENDOSTATIN, PEDF, and β-tubulin (as loading controls) in muscle extracts from GASTROC of TG and WT mice (n = 3).

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revascularization over time and serves as a good model for exploring the regulation of neoangiogenesis (Matsakas et al., 2012b). To determine the role of PGC1β in ischemic revascularization or neoangiogenesis, we applied unilateral hindlimb ischemia to both muscle-specific PGC1β TG and WT littermate mice. Next, we measured skeletal muscle revascularization in these mice using laser-Doppler flowmetry. We found that whereas ischemic revascularization restored blood perfusion in the WT TA within 15 days, this process was impaired in the TG TA (Figure 2C). The baseline muscle blood flow was comparable between the WT and TG mice (WT = 6.14 ± 0.53 versus TG = 7.1 ± 0.77 [ml/min/100 g tissue]). Also, the two genotypes showed comparable muscle capillary staining (Figure S4B). Furthermore, the ischemic TG TA cryosections stained for the endothelial marker CD31 showed a dramatic decrease in endothelial-cell staining compared with the ischemic WT TA (Figure 2D; quantified in Figures 2E and 2F), demonstrating less capillarization in the PGC1β-overexpressing muscles. Therefore, PGC1β inhibits ischemic neoangiogenesis, but does not affect developmental skeletal muscle vascularization.

We investigated the comprehensive effect of PGC1β at the transcription level by performing a microarray analysis of TA muscles from WT and muscle-specific PGC1β TG mice. We found that PGC1β differentially regulated a total of 3,627 genes in skeletal muscles, of which 1,594 genes were upregulated and 2,033 were downregulated (Figure S5A). The Gene-Ontology-based classification of PGC1β-regulated genes in biological pathways is shown in Figure 2G, including gene sets linked to mitochondrial function (216), intracellular signaling (157), and carbohydrate/lipid metabolism (146). More relevantly, the pathway-based analysis confirmed that PGC1β downregulates proangiogenic genes and upregulates antiangiogenic genes, creating a net antiangiogenic signal (Figure 2H). Interestingly, genes linked to VEGF cellular signaling were also downregulated by PGC1β (Figure S5B), further supporting the antiangiogenic role of PGC1β.

To corroborate our findings from the PGC1β TG mice and examine the role of endogenous PGC1β in neoangiogenesis, we asked whether deletion of PGC1β expression actually accelerates ischemic skeletal muscle revascularization. For this purpose, we applied unilateral hindlimb ischemia to PGC1β$^{-/-}$ and WT littermate mice and measured the revascularization. We found that muscle revascularization in response to ischemic insult was enhanced in the PGC1β$^{-/-}$ mice compared with the WT mice (Figure 3A). The baseline muscle blood flow was comparable between the WT and PGC1β$^{-/-}$ mice (WT = 5.96 ± 0.75 versus PGC1β$^{-/-}$ = 6.07 ± 0.59 [ml/min/100 g tissue]). Whole-mount imaging of ischemic PGC1β$^{-/-}$ and WT muscles of mice perfused with microfil dye showed that PGC1β$^{-/-}$ muscles had enhanced vascularization compared with the WT muscles (Figures 3B [whole muscle] and 3C [microscopic image]). Notably, antiangiogenic genes such as *Thbs1*, *Thbs2*, and *Vash1* were downregulated in the PGC1β$^{-/-}$ compared with the WT muscles (Figures 3D and 3E). However, the expression of proangiogenic genes was unaffected by PGC1β deletion (data not shown), suggesting that the enhanced ischemic neoangiogenesis in the PGC1β$^{-/-}$ mice might be primarily due to the repression of antiangiogenic genes. Collectively, these findings indicate that PGC1β limits ischemic muscle neoangiogenesis, inactivation of which can enhance the process of revascularization.

PGC1β Signaling in Endothelial Cells

Our findings in skeletal muscle raised the possibility that endothelial PGC1β might also encode an antiangiogenic gene program. To test this, we used SVEC4-10 mouse endothelial cells for in vitro studies and mouse dorsal aortic ring explants for ex vivo endothelial cell sprouting assays. Overexpression of PGC1β in murine endothelial SVEC4-10 cells (Figure 4A) inhibited the expression of proangiogenic genes (*Angpt1*, *Angpt2*, *Fgf1*, *Fgf2*, *Vegfc*, and *Vegfd*; Figure 4B) but induced antiangiogenic genes (*Thbs1*, *Thbs2*, and *Vash1*; Figure 4C). Accordingly, PGC1β inhibited cell migration in these endothelial cells (Figure 4D; quantified in Fig. 4E), a classical angiogenic process. Next, we investigated the effect of PGC1β on endothelial cells in a commonly used aortic-ring angiogenesis assay. Lentiviral overexpression of PGC1β induced expression of *Thsb1* and *Thsb2* (Figure 4G), and thus blocked the aortic sprouting (Figure 4F). Conversely, sprouting was enhanced in aortic-ring explants from PGC1β$^{-/-}$ compared with WT mice (Figure 4H; quantified in Fig. 4I). Moreover, this effect was rescued by overexpression of PGC1β in the mutant aortic rings (Figures 4H and 4I). In support of this, antiangiogenic gene *Thbs1* expression was decreased in aortic explants from PGC1β$^{-/-}$ mice and restored by PGC1β overexpression (Figure 4J; for data regarding PGC1β expression in these groups, see Figures 4G and 4J). Therefore, the effect of PGC1β on angiogenic gene expression might be cell autonomous and the cofactor might repress angiogenesis particularly via effects in both the ischemic muscle and endothelial cells.

PGC1β Regulates Antiangiogenic Genes via COUP TF-I

PGC1β is a transcriptional cofactor and is known to interact with several transcription factors and nuclear receptors. Therefore,

(C) Postischemic blood-flow recovery (ischemic to contralateral ratio) in TG (filled diamonds) and WT (open circles) mice (n = 6, mean ± SD). **p < 0.001 represents statistically significant differences (two-way ANOVA and Bonferonni's multiple-comparison test).
(D) Representative TA cryosections from TG and WT mice 14 days postischemia immunostained for capillary structures (n = 3). Scale, 100 μm.
(E) Quantification of vascularization presented as capillaries/myofiber in ischemic TA from TG and WT muscles (n = 3, mean ± SD). *p < 0.01 (unpaired Student's t test).
(F) Ratio of CD31-positive cells (ischemic to contralateral) in TA from TG and WT (n = 3). *p < 0.01 (unpaired Student's t test).
(G) Pie chart showing the major cellular pathways transcriptionally regulated by PGC1β (>2-fold) in the microarray analysis of TA from TG and WT mice (n = 3; p < 0.05).
(H) Up- or downregulated pro- and antiangiogenic genes from the microarray analysis (p < 0.05).
See also Figures S2–S5.

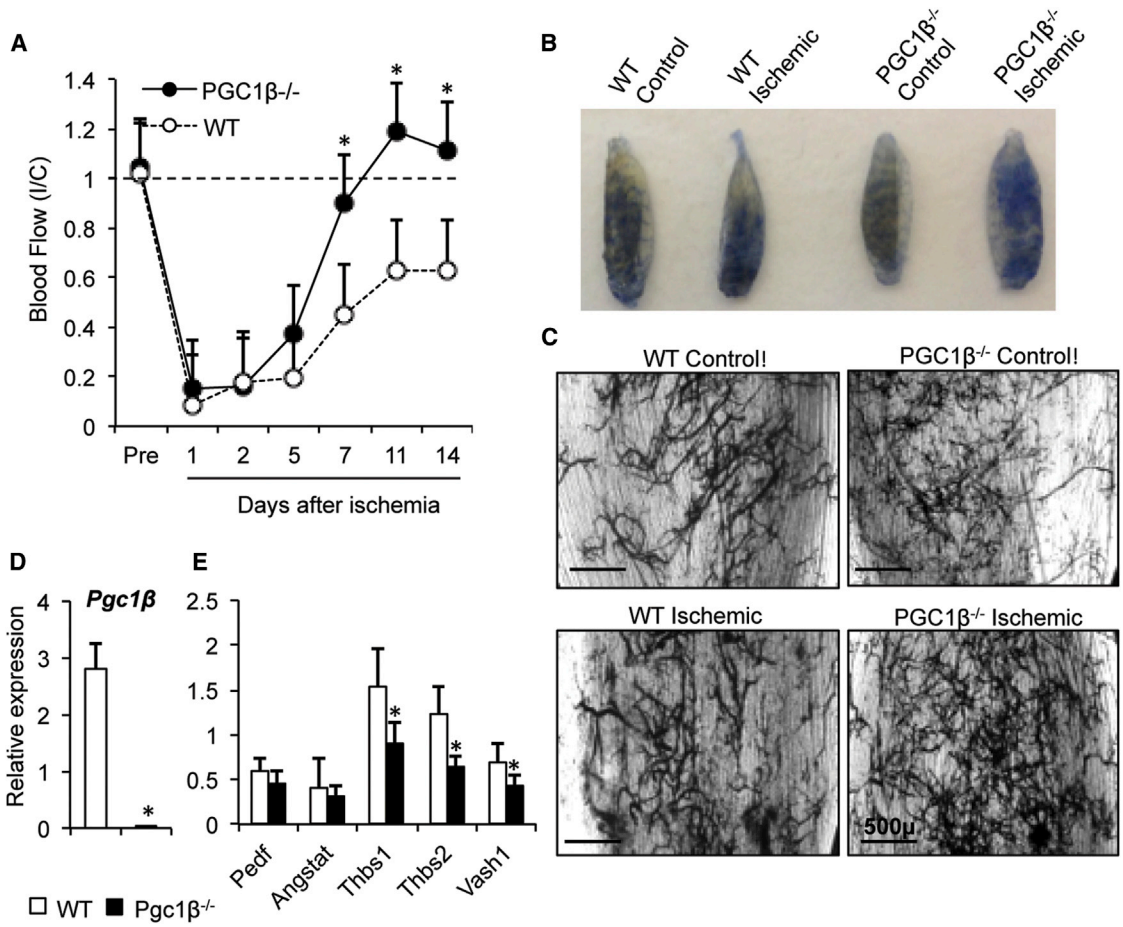

Figure 3. Loss of PGC1β Enhances Ischemic Revascularization

(A) Postischemic perfusion in PGC1β$^{-/-}$ and WT littermate mice (n = 6, mean ± SD). *p < 0.01 (two-way ANOVA and Bonferonni's multiple-comparison test).

(B) Representative images of TA muscles perfused with microfil dye to detect intact vasculature in PGC1β$^{-/-}$ and WT mice.

(C) Whole-mount image of microfilm perfused TA from PGC1β$^{-/-}$ and WT mice (n = 3). Scale, 500 μm.

(D and E) Relative mRNA expression of PGC1β (D) and antiangiogenic (E) genes in TA from PGC1β$^{-/-}$ and WT mice (n = 5, mean ± SD). *p < 0.05 (unpaired Student's t test).

PGC1β might regulate angiogenic genes by activating one or more transcriptional factors on different genes. To reveal the mechanism by which PGC1β regulates the antiangiogenic gene program, we examined the promoters and first intronic regions of the potent antiangiogenic factors Thbs1 and Thbs2. We targeted these two genes to focus more on the regulation of antiangiogenic genes by PGC1β. We found that both of these genes possess a consensus sequence for COUP-TF1 binding (5′-(A/G)G(G/T)TCA-3′) (Figures 5A and 5B). To gain further insight, we performed chromatin immunoprecipitation (ChIP) on sheared chromatin from C2C12 myotubes expressing histidine-tagged PGC1β (PGC1β-HIS) using an antihistidine antibody. We found PGC1β enrichment at the COUP TF-I binding sites in the Thbs1 (site −2,030) and Thbs2 (site −1,064) promoter regions (Figures 5C and 5D). Next, we tested whether PGC1β regulates *Thbs1* and *Thbs2* by activating COUP TF-I. In transient transfection experiments, PGC1β overexpression in C2C12 cells induced both *Thbs1* and *Thbs2*, an effect that was further enhanced in the presence of COUP-TFI (Figures 5E and 5F). To further support our findings, 2.7 kb of human THBS1 (hTHBS1) and 2.85 kb of mouse Thsb2 (mThsb2) genomic DNA fragments encompassing the COUP-TF1 binding sites were cloned 5′ to a luciferase reporter gene. In 293T cells, cotransfection of PGC1β with COUP-TF1 activated both THBS1 and THBS2 promoter activities compared to controls, whereas cotransfection of PGC1β with COUP-TFII did not activate either promoter. In reporter gene assays, both the Thbs1 and Thbs2 promoters were synergistically activated by PGC1β and COUP TF-I (Figures 5G and 5H). Interestingly, we also found that COUP-TFI (but not COUP-TFII) gene and protein expression was induced by PGC1β in the skeletal muscles isolated from PGC1β TG mice compared to the WT mice (Figures 5I–5K). PGC1β overexpression similarly induced COUP-TFI expression in C2C12 cells (Figure S6). Therefore, PGC1β might stimulate some of the antiangiogenic genes by inducing as well as coactivating the orphan nuclear receptor COUP-TFI.

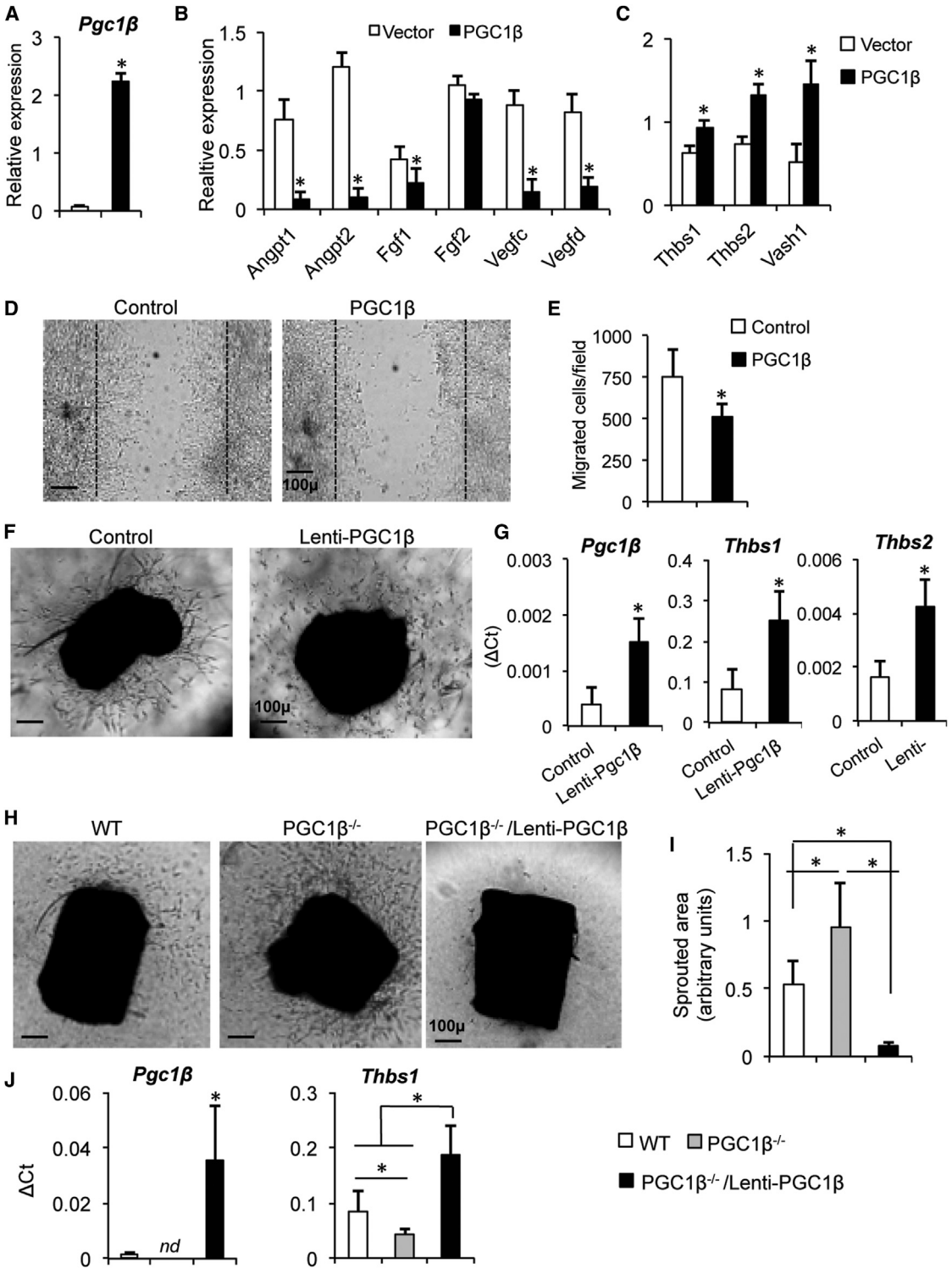

Figure 4. PGC1β Negatively Regulates Angiogenesis in Endothelial Cells

(A) PGC1β mRNA expression in empty vector (control) and PGC1β-overexpressing SVEC4-10 endothelial cells.

(B and C) Relative mRNA expression of pro- and antiangiogenic genes in SVEC4-10 cells overexpressing PGC1β and compared with control cells (n = 6, mean ± SD). *p < 0.01 (unpaired Student's t test).

(D and E) PGC1β-overexpressing SVEC4-10 cells are hypomigratory in a scratch assay.

(F) Dorsal aortic-ring assay in aortic explants infected with either PGC1β (lenti-PGC1β) or empty vector (control) lentivirus (n = 10).

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Hypoxia Represses PGC1β Expression

Hypoxia is a hallmark of ischemic diseases and activates a proangiogenic gene program to promote revascularization (Semenza, 2009). Because our results showed that PGC1β is an antiangiogenic regulator, we rationalized that hypoxia would downregulate PGC1β. Indeed, PGC1β gene expression was reduced in C2C12 muscle cells subjected to 6 hr of hypoxia compared to those in normoxia (Figure 6A). The same hypoxic conditions that repressed PGC1β robustly induced hallmark hypoxia-stimulated proangiogenic factors (e.g., *Vegfa*, *Vegfd*, *Nos2*, and *Fgf1*) in the C2C12 cells (Figure 6B). Additionally, we investigated whether the hypoxia-mimetic drug dimethyloxaloylglycine (DMOG) could also repress PGC1β expression. Treatment of C2C12 cells with DMOG (1 mM) repressed PGC1β expression and simultaneously increased *Vegfa*, *Vegfd*, *Nos2*, and *Fgf1* expression (Figures 6C and 6D). Similar to what was observed for gene expression, hypoxia also reduced PGC1β protein expression under conditions that stimulated the expression of proangiogenic factors (Figure 6E). Similar to the case with hypoxia, PGC1β expression was also inhibited 4 days after ischemia in the skeletal muscle (Figure 6F). Interestingly, hypoxia also repressed PGC1β expression in endothelial cells (HUVECs and SVEC4-10; Figures S7A and S7B). Therefore, our results unequivocally show that PGC1β expression is inhibited by hypoxia, a hypoxia-mimetic drug, and ischemia, which is congruous with the antiangiogenic role of PGC1β.

Hypoxic Repression of PGC1β Is Mediated by HIF Signaling

HIF is a central hypoxic regulator that stimulates a robust proangiogenic gene program (Semenza, 2009, 2012). Therefore, we hypothesized that HIF itself mediates hypoxic repression of PGC1β. To test this hypothesis, we investigated whether the hypoxic repression of PGC1β is lost in cells devoid of HIF signaling. To do so, we used cells lacking HIF1B (also known as aryl hydrocarbon receptor nuclear translocator [ARNT]; ARNT$^-$ cells, ATCC #CRL-2717) as well as control cells that express HIF1B/ARNT (ARNT$^+$ cells, ATCC# CRL-2712) (Wood et al., 1996). HIF1B/ARNT is an obligatory heterodimeric partner in the HIF signaling complex, without which the cells are devoid of HIF signaling (Semenza, 2012; Wood et al., 1996). We subjected both the ARNT$^+$ and ARNT$^-$ cells to hypoxia for 6 hr and then measured the gene expression of PGC1β as well as the HIF target genes *Vegfa* and *Nos2*. We found that, similar to what was observed in muscle cells, hypoxia repressed PGC1β expression in the control ARNT$^+$ cells. Not only was this effect lost in ARNT$^-$ cells, but also PGC1β expression was surprisingly induced under hypoxic conditions in these cells (Figure 7A). We showed that expression of the HIF target genes *Vegfa* and *Nos2* was induced in ARNT$^+$ cells by hypoxia, but this effect was lost in ARNT$^-$ cells, confirming the lack of HIF signaling (Figure 7B).

We further explored the involvement of HIF in hypoxic repression of PGC1β in muscle cells. We overexpressed constitutively active HIF1A (HIF1A-CA) or control vector in C2C12 cells and subjected the infected cells to 6 hr of hypoxia or normoxia. In control cells, PGC1β was repressed by hypoxia. PGC1β gene expression was repressed by HIF1A-CA even in normoxia. The hypoxic repression of PGC1β was further exaggerated in muscle cells expressing HIF1A-CA (Figure 7C). At the protein level, HIF1A-CA repressed PGC1β expression similarly to hypoxic repression of the cofactor (Figure 7D; quantified in Figure 7E).

Next, we determined whether HIF represses PGC1β transcription by direct promoter occupancy on the *Pgc1β* gene. For this purpose, we searched the *Pgc1β* gene promoter and first intronic region for the presence of the HIF response element (HRE) consensus sequence ((A/G)CGTG(C/G)). We found at least one binding site in the promoter region and six sites in the first intron (Figure 7F). Accordingly, we performed ChIP for HIF occupancy on these HRE sites in the endogenous *Pgc1β* gene. ChIP was performed with C2C12 cells using HA-tagged HIF1A under hypoxia and normoxia. Chromatin was precipitated with anti-HA followed by PCR and qPCR using primers flanking the HRE sites. We observed that HIF1A occupied the *Pgc1β* gene at the +1,005 bp position in the first intron with enhanced occupancy under hypoxic conditions (Figures 7G and 7H). As a positive control, we show Vegfa gene occupancy by HIF1A under normoxia and hypoxia. To determine whether HIF1A regulates the PGC1β promoter activity, 500 bp and 450 bp fragments of DNA surrounded by HIF1A-binding sites from the PGC1β first intron corresponding to positions +1,005 and +1,929, respectively, were cloned 5′ to a luciferase reporter gene. First, the 293T cells were cotransfected with the PGC1β luciferase construct (+1,005) and plasmids encoding for HIF1A or HIF1A-CA. Whereas HIF1A-CA was sufficient to suppress the PGC1β promoter activity under both normoxia and hypoxia, HIF1A blocked the promoter activity only under hypoxic conditions (Figure 7I). In contrast, the PGC1β luciferase construct with the +1,929 HIF1A site was not repressed by HIF1A in either normoxia or hypoxia (Figure 7J).

Collectively, our findings demonstrate that hypoxia (a proangiogenic stimuli) represses PGC1β in a HIF-dependent fashion, and provide a molecular basis to explain how pro- and antiangiogenic genes might be balanced in hypoxia or ischemia to promote neoangiogenesis (Figure 7K).

DISCUSSION

Loss of skeletal muscle vasculature leading to limb amputation is a common complication in metabolic and cardiovascular diseases. Although muscle angiogenesis is governed by a balance between pro- and antiangiogenic factors, most studies have focused on the molecular regulators of proangiogenic factors. How the antiangiogenic program is encoded is poorly defined.

(G) mRNA expression of PGC1β, Thbs1, and Thbs2 in mouse aortic explants at day 7 (n = 4, mean ± SD). *p < 0.01 (unpaired Student's t test).
(H) Aortic-ring assay in WT, PGC1β$^{-/-}$, and PGC1β-infected PGC1β$^{-/-}$ aortic explants (n = 7).
(I) Quantification of the sprouted area from the aortic rings shown in (H).
(J) mRNA expression of Pgc1β and Thbs1 in mouse aortic explants at day 7 (n = 3, mean ± SD). *p < 0.05 (unpaired Student's t test); nd, not detectable (Ct ≥ 35).
Where indicated, scale bar represents 100 μm.

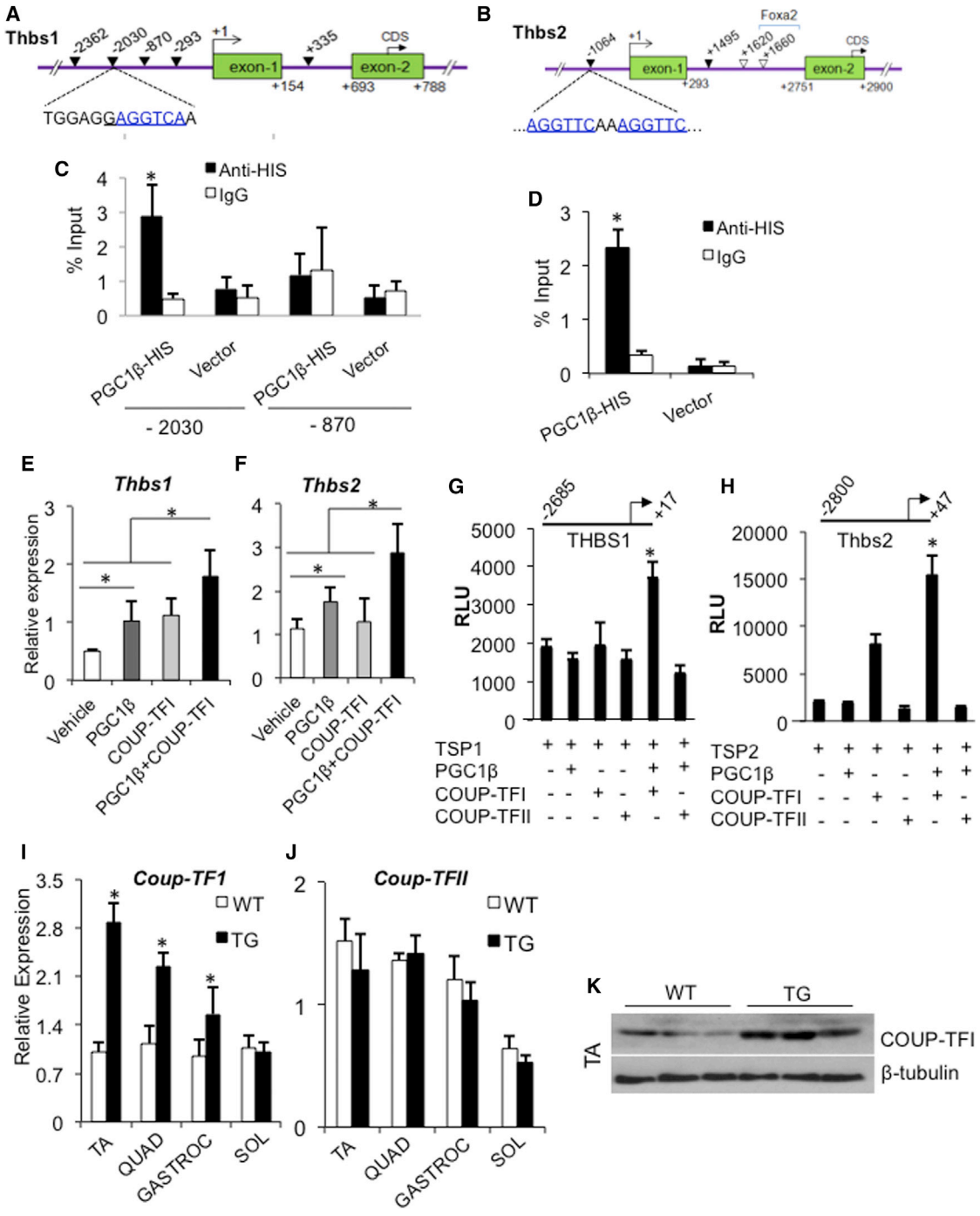

Figure 5. PGC1β Regulates the Expression of Thbs1 and Thbs2 by Coactivating COUP-TFI

(A and B) COUP-TF responsive elements (COUP-RE) in the promoter and first intron of THBS1 (A) and THBS2 (B) genes. Underlined nucleotides show the direct repeat (DR) of the COUP-TF binding sequence (ATTGGC).

(C and D) ChIP qPCR for the occupancy of HIS-tagged PGC1β at COUP-TF binding regions on the THBS1 promoter (−2,030 and −870 sites) and THBS2 gene promoter at the −1,064 site (n = 3, mean ± SD). *$p < 0.01$ (unpaired Student's t test).

(E and F) Cotransfection of PGC1β and COUP-TFI is able to induce both Thbs1 and Thbs2 in C2C12 myotubes (n = 4, mean ± SD). **$p < 0.05$ (unpaired Student's t test).

(G and H) Reporter assays for mouse THBS2 (mTHBS2) or human THBS1 (hTHBS1) promoter activation by PGC1β, COUP-TFI, COUP-TFII, PGC1β + COUP-TFI, and PGC1β + COUP-TFII (n = 6, mean ± SD). **$p < 0.001$ (unpaired Student's t test).

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We show that the nuclear receptor coactivator PGC1β activates an antiangiogenic program in both muscle and endothelial cells by inhibiting proangiogenic genes and stimulating antiangiogenic genes. Consequently, PGC1β delays revascularization of the skeletal muscle in ischemia. The PGC1β antiangiogenic gene program is partly dependent on activation of the orphan nuclear receptor COUP-TFI. Interestingly, PGC1β is downregulated in response to ischemia, hypoxia, or a hypoxia-mimetic drug in a HIF1A-dependent fashion. Therefore, PGC1β is a HIF1A-regulated antiangiogenic factor that could be targeted for therapeutic angiogenesis.

The previously reported proangiogenic function of PGC1α in controlling muscle vasculature by stimulating Vegfa (Arany et al., 2008; Chinsomboon et al., 2009) prompted us to ask whether PGC1β also regulates angiogenesis. We found that in contrast to PGC1α, PGC1β represses proangiogenic genes in muscle cells, most of which encode secretory angiokines that promote angiogenesis, such as *Fgf1*, *Fgf2*, *Vegfc*, and *Vegfd*. In addition, PGC1β transcriptionally stimulates the expression of antiangiogenic secretory factors that repress angiogenesis, such as *Thbs1*, *Thbs2*, *Angstat*, and *Vash1*. Accordingly, muscle lysates from TG muscles overexpressing PGC1β blocked tube formation in HUVECs, demonstrating a net antiangiogenic signal emanating from PGC1β-activated muscles. It was recently shown that PGC1β overexpression in muscle cells induced Vegfa expression (Rowe et al., 2011). However, a comprehensive profiling of angiogenic factors was not performed in that study. We observed a similar induction of Vegfa in C2C12 muscle cells overexpressing PGC1β. However, in our studies, overexpression of PGC1β in rodent muscles neither induced Vegfa isoforms nor changed the basal vasculature or blood flow in the skeletal muscles. A global gene-expression analysis generally revealed a repression of proangiogenic genes and a stimulation of antiangiogenic genes by PGC1β in the skeletal muscles. Indeed, overexpression of PGC1β in the skeletal muscles inhibited revascularization of the muscles in response to ischemic attack. The antiangiogenic role of PGC1β, specifically the endogenously expressed coactivator, was further confirmed by our observation that ischemic revascularization of the skeletal muscle was enhanced in PGC1β$^{-/-}$ compared with WT mice. Therefore, PGC1β drives a net antiangiogenic gene program that involves inhibition of proangiogenic genes and stimulation of antiangiogenic genes, and blocks neoangiogenesis and revascularization in the skeletal muscles.

We found that the antiangiogenic gene effects of PGC1β are cell autonomous. Similarly to muscle cells, PGC1β activates an antiangiogenic program in endothelial cells that leads to blockade of angiogenic phenomena, as revealed by in vitro angiogenesis assays for endothelial cell migration and dorsal aortic-ring sprouting. In contrast, the angiogenic effects of PGC1α seem to be cell-type specific. Whereas PGC1α activates angiogenesis in muscle through paracrine secretion of proangiogenic factors such as Vegfa (Arany et al., 2008), it inhibits angiogenesis by activating notch signaling and blunting the effects of proangiogenic factors in endothelial cells (Sawada et al., 2014). Activation of notch signaling in endothelial cells is responsible for limiting angiogenesis and maintaining the cells in quiescence (Guarani et al., 2011). Whether PGC1β activates notch signaling in endothelial cells is unknown and will be interesting to examine in the future. Likewise, the regulation of smooth muscle cells by PGCs and how this regulation contributes to vascularization will be crucial to investigate.

PGC1β orchestrates gene expression by activating multiple transcriptional factors or nuclear receptors (Finck and Kelly, 2006). While it can be envisioned that a similar mechanism is at play in the regulation of angiogenesis by PGC1β, we focused on the regulation of the potent antiangiogenic factors Thbs1 and Thsb2 to identify at least one transcriptional factor involved in PGC1β effects. We chose to focus on these antiangiogenic genes because of the need to improve our understanding of how antiangiogenic factors are regulated in the muscle. We observed that both of these genes have orphan nuclear receptor COUP-TFI binding sites in the promoter and first intronic regions. We found that PGC1β-dependent regulation of both of these genes was enhanced in the presence of the orphan nuclear receptor COUP-TFI, and PGC1β binds at the COUP-TFI binding site in the promoters of Thbs1 and Thbs2. Interestingly, COUP-TFI was itself induced by PGC1β overexpression in the skeletal muscles, potentially suggesting a dual effect on COUP-TF1 in regulating antiangiogenic genes. Interestingly, a related orphan nuclear receptor, COUP-TFII, is known to regulate angiogenesis (Pereira et al., 1999; Qin et al., 2010). However, PGC1β neither increased COUP-TFII expression nor regulated Thbs1 and Thbs2 through COUP-TFII. Although not much is known regarding how COUP-TF1 regulates angiogenesis, our results preliminarily suggest that COUP-TFI might be a PGC1β-activated antiangiogenic factor.

In concert with the antiangiogenic role of PGC1β, we found that PGC1β is downregulated by hypoxia, the hypoxia-mimetic drug DMOG, and ischemia. Since all of these conditions activate HIF1A, we investigated whether HIF1A is responsible for blocking PGC1β expression. The inhibition of PGC1β expression by hypoxia is lost in cells lacking HIF1A signaling, suggesting a direct regulation of the coactivator expression by HIF1A. Indeed, HIF1A inhibits PGC1β gene expression by directly binding at the first intronic site in the PGC1β gene. Notably, expression of PGC1β is decreased in VHL-dependent renal carcinoma cells, in which HIF1 activity is high (Zhang et al., 2007). In these cells, PGC1β expression is controlled by C-MYC, which in turn is downregulated or degraded by HIF1A. Direct regulation of PGC1β by HIF1 was not examined in this study. Nevertheless, downregulation of PGC1β is a component of the hypoxic or HIF1A activation response, which is in agreement with the antiangiogenic role of the coactivator.

Why PGC1β-driven antiangiogenic genes do not affect the vascular supply in normoxic muscle is not clear. In terms of fiber

(I and J) Relative expression of COUP-TFI (I) and COUP-TFII (J) in various muscle beds from TG and WT mice (n = 5, mean ± SD). **$p < 0.01$ (unpaired Student's t test).
(K) Representative western blots for of COUP-TFI and β-tubulin (as loading control) in TA from TG and WT mice (n = 3).
See also Figure S6.

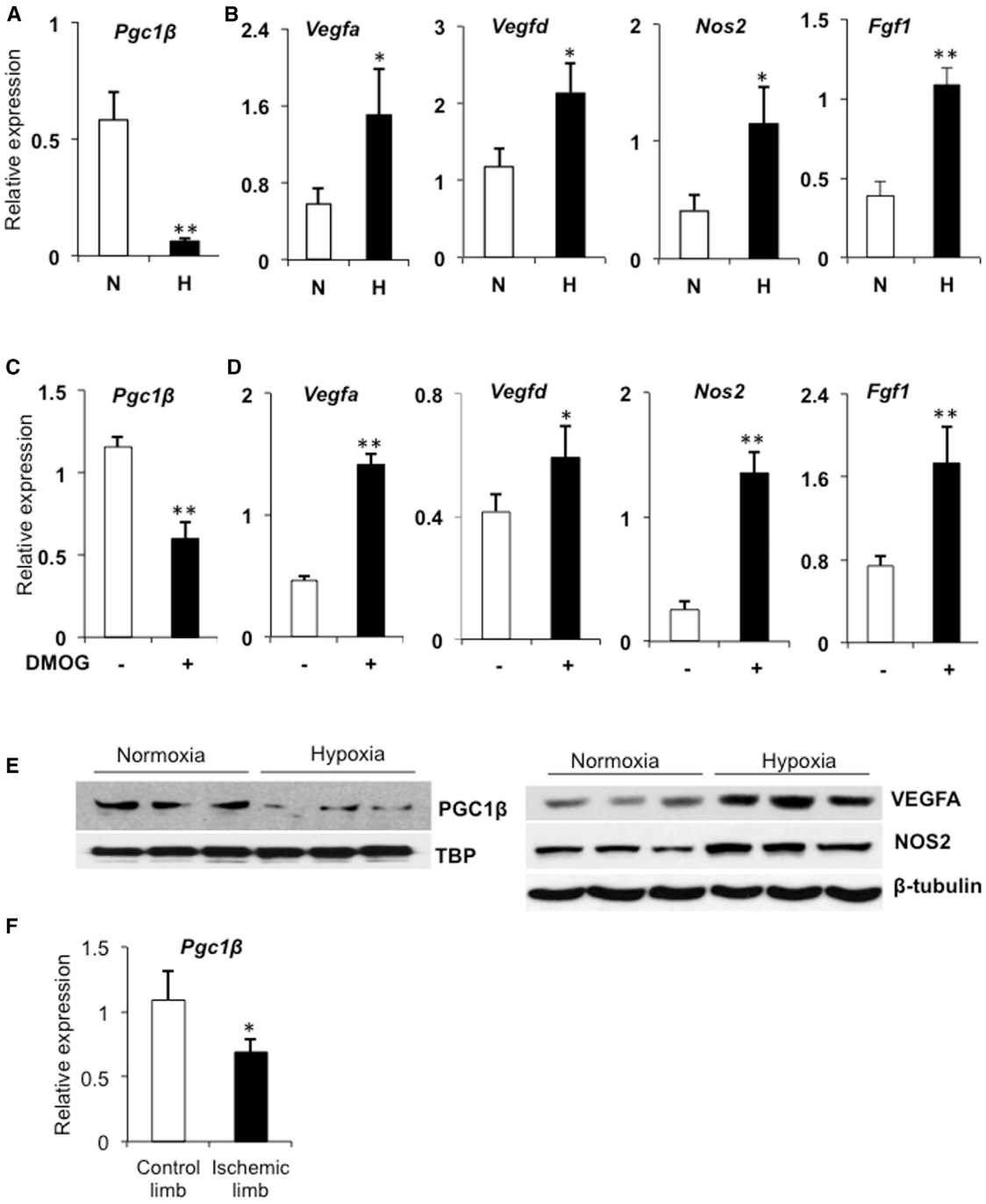

Figure 6. Regulation of PGC1β by Hypoxia

(A and B) PGC1β (A) and proangiogenic (B) gene expression in C2C12 myotubes subjected to hypoxia (H) or normoxia (N) for 6 hr (n = 6).

(C and D) PGC1β (C) and proangiogenic (D) gene expression in C2C12 myotubes treated for 24 hr with the hypoxia-mimetic drug DMOG (1 mM; n = 5). In (A)–(D), data are presented as mean ± SD. *p < 0.01, **p < 0.0001 (unpaired Student's t test).

(E) Representative western blots of PGC1β and TBP (as loading controls) in nuclear extracts and VEGFA, NOS2, and β-Tubulin (as loading controls) in cytoplasmic extracts from C2C12 myotubes subjected to either normoxia or hypoxia for 6 hr.

(F) mRNA level of PGC1β in control and 4-day postischemic quadriceps (n = 4, mean ± SD). *p < 0.05 (unpaired Student's t test).

See also Figures S7A and S7B.

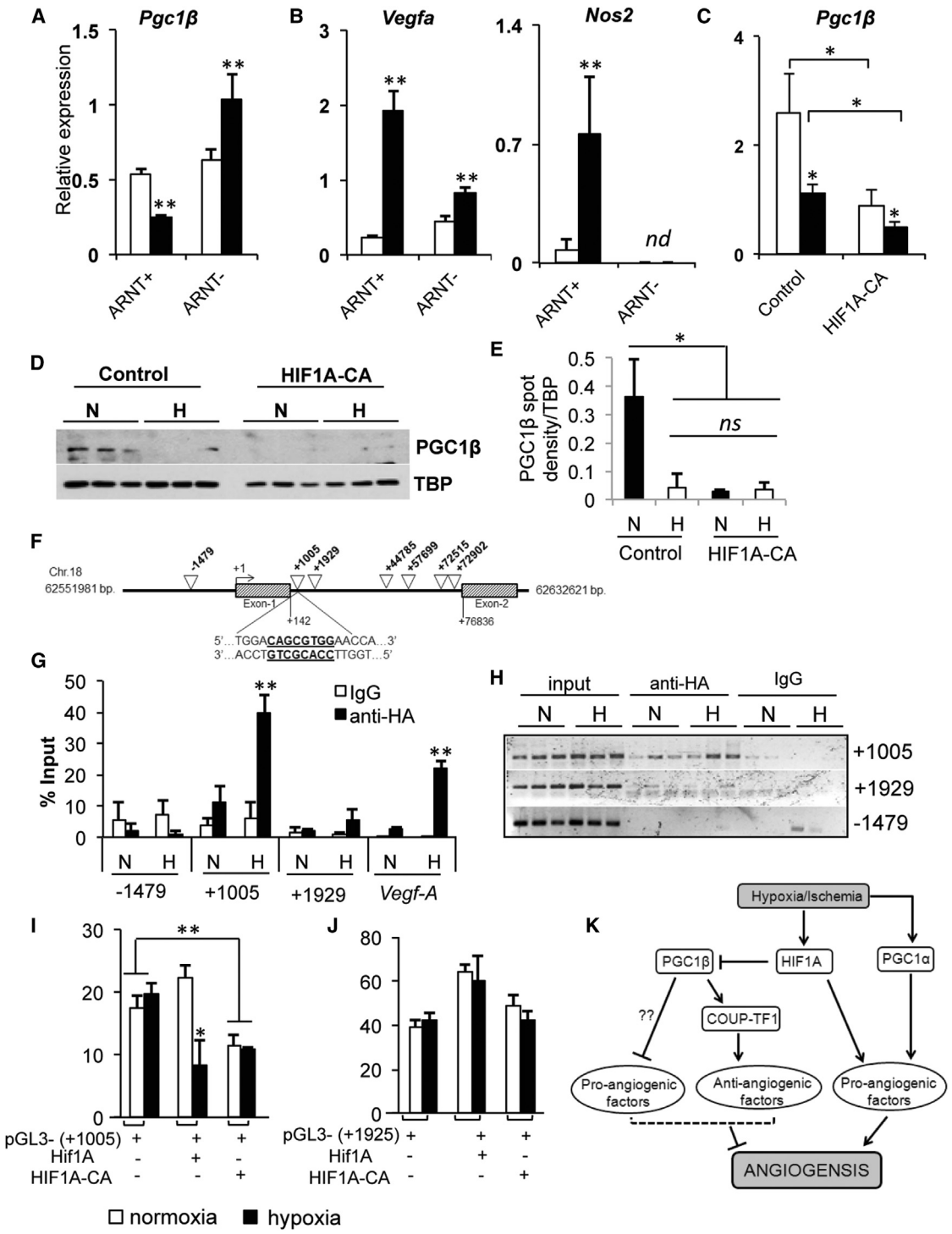

Figure 7. Hypoxic Repression of PGC1β Is Mediated by HIF1A

(A and B) Expression of PGC1β (A) and the HIF1 target genes Vegfa and NOS2 (B) in ARNT^{+} and ARNT^{-} cells subjected to normoxia (open bar) or hypoxia (closed bar) for 6 hr (n = 5, mean ± SD). *p < 0.01 and **p < 0.0001 (unpaired Student's t test); nd, not detectable (Ct ≥ 35).

(C) Expression of PGC1β in control or constitutively active human HIF1A (HIF1A-CA)-overexpressing C2C12 cells subjected to normoxia or hypoxia for 6 hr (n = 5, mean ± SD). *p < 0.01 and **p < 0.0001 (unpaired Student's t test).

(D and E) Representative immunoblots (D) and quantification (E) of PGC1β and TBP (as loading control) protein expression in nuclear extracts from C2C12 myotubes as in (C). Error bars indicate mean ± SD; **p < 0.001 (unpaired Student's t test); ns, not significant.

(F) HREs in the promoter and first intron of the *PGC1β* gene.

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type and metabolic capacity, PGC1β overexpression in muscle increases type IIX myofibers, oxidative capacity, mitochondrial biogenesis, and exercise tolerance (Arany et al., 2007), which presumably should be matched by an increased vascular supply. This is at least the case with other metabolic regulators such as PGC1α and ERRγ, which coregulate aerobic metabolism and angiogenesis in the muscle (Arany et al., 2008; Lin et al., 2002; Narkar et al., 2011). However, our data suggest that PGC1β does not affect the muscle vascular supply in normoxia. How might existing vasculature support PGC1β-mediated metabolic changes? One possibility is that a compensatory vasodilation occurs in the muscle microcirculation to meet metabolic demands. However, we were unable to detect any change in basal blood flow in PGC1β-overexpressing or null skeletal muscles. Notably, exercise, which enhances both muscle oxidative capacity and angiogenesis, only induces coregulators of these phenomena, such as PGC1α, ERRα, and ERRγ, but not PGC1β (Akimoto et al., 2005; Cartoni et al., 2005; Matsakas et al., 2012a). On the other hand, as we have shown, PGC1β is downregulated in hypoxia or ischemia, where lack of oxygen or nutrients would necessitate silencing of oxidative and antiangiogenic gene programs such as those driven by PGC1β (see Figure S7C). Along the same lines, under physiological conditions, PGC1β may serve as a "brake" to prevent uncontrolled and aberrant angiogenesis, which needs to be removed during vascular insufficiency.

Peripheral vascular disease, stroke, and cardiac myopathies are linked to insufficient tissue vascular supply and can benefit from enhanced neoangiogenesis (Baumgartner et al., 2005; Chi et al., 2011; Varu et al., 2010). In contrast, diabetic retinopathy is associated with uncontrolled and aberrant vessel formation, and could be controlled by inhibition of neoangiogenesis (Chung et al., 2010; Costa and Soares, 2013). Despite this prevalent role of angiogenesis in various diseases, molecular regulation of pathological angiogenesis (beyond regulators such as HIF1A) and particularly of the antiangiogenic program remains poorly understood. Here, we have demonstrated that PGC1β drives a net antiangiogenic program by repressing stimulatory angiokines and inducing inhibitory angiokines, which specifically limit ischemic neoangiogenesis. The selectivity of PGC1β-mediated angiostatic effects on ischemia has implications for designing therapies for angiogenesis-linked diseases of muscle and other organs.

EXPERIMENTAL PROCEDURES

All experiments are described in detail in the Supplemental Experimental Procedures.

Animal Husbandry

The mice used in this study were maintained in the vivarium at the Brown Foundation Institute of Molecular Medicine for the Prevention of Human Diseases (UT Medical School) under standard environmental conditions (20–22°C, 12 hr light/12 hr dark cycle) and provided tap water ad libitum. Muscle-specific PGC1β TG male mice (12–14 weeks old) and whole-body PGC1β knockout male mice (16–18 weeks old) were used in the experiments. The muscle-specific PGC1β TG mice were generated in our laboratory on the C57Bl/6J background strain. The whole-body PGC1β knockout mice were obtained from Dr. Evans (Salk Institute). The generation of these PGC1β$^{-/-}$ mice was described previously (Sonoda et al., 2007). In our laboratory, the mice were backcrossed for two generations to the C57Bl/6J background strain. For all rodent experiments, littermate WT mice were always used as controls. The animals were maintained and treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals and with the approval of the Animal Welfare Committee of the University of Texas Health Science Center, Houston.

Cell Culture

C2C12 cells, SVEC4-10 endothelial cells, 293T cells, HUVECs, ARNT^{+} cells, and ARNT^{-} cells were cultured, transfected/infected, and treated as described in the Supplemental Experimental Procedures and as indicated in the figure legends.

Generation of Muscle-Specific PGC1β TG Mice

Muscle-specific PGC1β TG mice were generated using muscle-specific human alpha skeletal actin promoter, mouse PGC1β cDNA, and standard TG techniques as described in the Supplemental Experimental Procedures.

Gene Expression

Total RNA was prepared using the RNeasy Mini Kit (QIAGEN) and analyzed by qPCR using the Applied Biosystems SYBR Green PCR Master Mix with an ABI-7900 cycler (Applied Biosystems). Lists of the qPCR primer sets and sequences used are provided in the Supplemental Experimental Procedures.

PCR-Based Gene Array

PCR arrays were carried out using the mouse Angiogenesis PCR Array (catalog No. PAMM-024; SA Biosciences). The array profiles the expression of 84 genes involved in angiogenesis. PCR array data were analyzed using the online data analysis software from the manufacturer's website.

Protein Extraction and Immunoblotting

The procedures used for nuclear, cytoplasmic, and whole-cell lysate protein extraction, as well as western blotting and the antibodies used, are described in the Supplemental Experimental Procedures.

ChIP Assay

ChIP was performed using ChIP-IT Express (Active Motif) according to the manufacturer's instructions. Details regarding the antibodies used, as well as PCR and qPCR-based detection of binding sites, are provided in the Supplemental Experimental Procedures.

Hindlimb Ischemia, Tissue Collection, and Laser-Doppler Blood Flowmetry

Hindlimb ischemia was induced by unilateral femoral occlusion, and blood flow was measured by laser-Doppler blood flowmetry as described in the Supplemental Experimental Procedures. TA muscles were harvested at the indicated time points after induction of ischemia and processed.

Immunohistochemistry

Immunostaining of skeletal muscle capillaries was performed using CD31 staining in cryosections as described in the Supplemental Experimental Procedures.

Microfil Perfusion and Imaging

Whole-mount vascular imaging of the TA vasculature was performed after microfil angiography as detailed in the Supplemental Experimental Procedures.

(G and H) ChIP-qPCR (G) and ChIP-PCR (H) for the occupancy of HA-tagged HIF1A on *PGC1β* gene (n = 3, mean ± SD). **p < 0.001 (unpaired Student's t test).
(I and J) Luciferase (Luc) activity in 293T cells transfected with the PGC1β first intronic region having either (I) +1,005 HRE or (J) +1,925 HRE or control promoter constructs in pGL3B plasmid (n = 5, mean ± SD). **p < 0.001 (one-way ANOVA).
(K) Model illustrating that PGC1β is a HIF1-regulated transcriptional coregulator that encodes an antiangiogenic gene program and inhibits neoangiogenesis.

In Vitro Angiogenesis Assays

In vivo angiogenesis assays, including tube-formation, scratch-migration, and aortic-ring assays, were performed using standard techniques and are detailed in the Supplemental Experimental Procedures.

Microarray Analysis

Genome-wide gene-expression analysis was performed on RNA isolated from TA muscles in WT and TG mice using Illumina Sentrix Beadchip Array Mouse WG-6.v2 arrays. Arrays were scanned with the BeadArray Reader (Illumina). Data were analyzed using GenomeStudio software (Illumina). Clustering and pathway analyses were performed with GenomeStudio and Ingenuity Pathway Analysis (Ingenuity Systems) software, respectively.

Additional experimental procedures are described in the Supplemental Experimental Procedures. Complete lists of the primers and antibodies used are provided in Tables S2–S4.

Statistical Analysis

Data are shown as the mean ± SD. For comparison between two groups, statistical analysis was performed by unpaired Student's t test. For ischemic reperfusion, data were analyzed by two-way ANOVA and Bonferroni's multiple-comparison test. The differences were considered statistically significant at $p < 0.05$.

ACCESSION NUMBERS

Microarray data have been deposited to the NCBI Gene Expression Omnibus under accession number GSE58699.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and four tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.06.040.

AUTHOR CONTRIBUTIONS

V.Y. and V.A.N. designed the experiments, analyzed and interpreted the data, and wrote the manuscript. V.Y., A.M., and S.L. conducted the experiments.

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REFERENCES

Akimoto, T., Pohnert, S.C., Li, P., Zhang, M., Gumbs, C., Rosenberg, P.B., Williams, R.S., and Yan, Z. (2005). Exercise stimulates Pgc-1alpha transcription in skeletal muscle through activation of the p38 MAPK pathway. J. Biol. Chem. *280*, 19587–19593.

Arany, Z., Lebrasseur, N., Morris, C., Smith, E., Yang, W., Ma, Y., Chin, S., and Spiegelman, B.M. (2007). The transcriptional coactivator PGC-1beta drives the formation of oxidative type IIX fibers in skeletal muscle. Cell Metab. *5*, 35–46.

Arany, Z., Foo, S.Y., Ma, Y., Ruas, J.L., Bommi-Reddy, A., Girnun, G., Cooper, M., Laznik, D., Chinsomboon, J., Rangwala, S.M., et al. (2008). HIF-independent regulation of VEGF and angiogenesis by the transcriptional coactivator PGC-1alpha. Nature *451*, 1008–1012.

Baumgartner, I., Schainfeld, R., and Graziani, L. (2005). Management of peripheral vascular disease. Annu. Rev. Med. *56*, 249–272.

Cao, Y., Hong, A., Schulten, H., and Post, M.J. (2005). Update on therapeutic neovascularization. Cardiovasc. Res. *65*, 639–648.

Carmeliet, P., and Jain, R.K. (2011). Molecular mechanisms and clinical applications of angiogenesis. Nature *473*, 298–307.

Cartoni, R., Léger, B., Hock, M.B., Praz, M., Crettenand, A., Pich, S., Ziltener, J.L., Luthi, F., Dériaz, O., Zorzano, A., et al. (2005). Mitofusins 1/2 and ERRalpha expression are increased in human skeletal muscle after physical exercise. J. Physiol. *567*, 349–358.

Chi, Y.W., Osinbowale, O., and Milani, R. (2011). Genetic association studies in peripheral arterial disease. J. La. State Med. Soc. *163*, 30–34, 36–37, 39.

Chinsomboon, J., Ruas, J., Gupta, R.K., Thom, R., Shoag, J., Rowe, G.C., Sawada, N., Raghuram, S., and Arany, Z. (2009). The transcriptional coactivator PGC-1alpha mediates exercise-induced angiogenesis in skeletal muscle. Proc. Natl. Acad. Sci. USA *106*, 21401–21406.

Chung, A.S., Lee, J., and Ferrara, N. (2010). Targeting the tumour vasculature: insights from physiological angiogenesis. Nat. Rev. Cancer *10*, 505–514.

Costa, P.Z., and Soares, R. (2013). Neovascularization in diabetes and its complications. Unraveling the angiogenic paradox. Life Sci. *92*, 1037–1045.

Finck, B.N., and Kelly, D.P. (2006). PGC-1 coactivators: inducible regulators of energy metabolism in health and disease. J. Clin. Invest. *116*, 615–622.

Gaudel, C., Schwartz, C., Giordano, C., Abumrad, N.A., and Grimaldi, P.A. (2008). Pharmacological activation of PPARbeta promotes rapid and calcineurin-dependent fiber remodeling and angiogenesis in mouse skeletal muscle. Am. J. Physiol. Endocrinol. Metab. *295*, E297–E304.

Guarani, V., Deflorian, G., Franco, C.A., Krüger, M., Phng, L.K., Bentley, K., Toussaint, L., Dequiedt, F., Mostoslavsky, R., Schmidt, M.H., et al. (2011). Acetylation-dependent regulation of endothelial Notch signalling by the SIRT1 deacetylase. Nature *473*, 234–238.

Han, J.K., Kim, H.L., Jeon, K.H., Choi, Y.E., Lee, H.S., Kwon, Y.W., Jang, J.J., Cho, H.J., Kang, H.J., Oh, B.H., et al. (2013). Peroxisome proliferator-activated receptor-δ activates endothelial progenitor cells to induce angio-myogenesis through matrix metallo-proteinase-9-mediated insulin-like growth factor-1 paracrine networks. Eur. Heart J. *34*, 1755–1765.

Kivelä, R., Silvennoinen, M., Touvra, A.M., Lehti, T.M., Kainulainen, H., and Vihko, V. (2006). Effects of experimental type 1 diabetes and exercise training on angiogenic gene expression and capillarization in skeletal muscle. FASEB J. *20*, 1570–1572.

Lawler, P.R., and Lawler, J. (2012). Molecular basis for the regulation of angiogenesis by thrombospondin-1 and -2. Cold Spring Harb Perspect Med *2*, a006627.

Lekas, M., Lekas, P., Latter, D.A., Kutryk, M.B., and Stewart, D.J. (2006). Growth factor-induced therapeutic neovascularization for ischaemic vascular disease: time for a re-evaluation? Curr. Opin. Cardiol. *21*, 376–384.

Lin, J., Wu, H., Tarr, P.T., Zhang, C.Y., Wu, Z., Boss, O., Michael, L.F., Puigserver, P., Isotani, E., Olson, E.N., et al. (2002). Transcriptional co-activator PGC-1 alpha drives the formation of slow-twitch muscle fibres. Nature *418*, 797–801.

Matsakas, A., Macharia, R., Otto, A., Elashry, M.I., Mouisel, E., Romanello, V., Sartori, R., Amthor, H., Sandri, M., Narkar, V., and Patel, K. (2012a). Exercise training attenuates the hypermuscular phenotype and restores skeletal muscle function in the myostatin null mouse. Exp. Physiol. *97*, 125–140.

Matsakas, A., Yadav, V., Lorca, S., Evans, R.M., and Narkar, V.A. (2012b). Revascularization of ischemic skeletal muscle by estrogen-related receptor-γ. Circ. Res. *110*, 1087–1096.

Milkiewicz, M., Pugh, C.W., and Egginton, S. (2004). Inhibition of endogenous HIF inactivation induces angiogenesis in ischaemic skeletal muscles of mice. J. Physiol. *560*, 21–26.

Muscat, G.E., and Kedes, L. (1987). Multiple 5′-flanking regions of the human alpha-skeletal actin gene synergistically modulate muscle-specific expression. Mol. Cell. Biol. *7*, 4089–4099.

Narkar, V.A., Fan, W., Downes, M., Yu, R.T., Jonker, J.W., Alaynick, W.A., Banayo, E., Karunasiri, M.S., Lorca, S., and Evans, R.M. (2011). Exercise and PGC-1α-independent synchronization of type I muscle metabolism and vasculature by ERRγ. Cell Metab. *13*, 283–293.

O'Hagan, K.A., Cocchiglia, S., Zhdanov, A.V., Tambuwala, M.M., Cummins, E.P., Monfared, M., Agbor, T.A., Garvey, J.F., Papkovsky, D.B., Taylor, C.T., and Allan, B.B. (2009). PGC-1alpha is coupled to HIF-1alpha-dependent gene expression by increasing mitochondrial oxygen consumption in skeletal muscle cells. Proc. Natl. Acad. Sci. USA *106*, 2188–2193.

Olfert, I.M., and Birot, O. (2011). Importance of anti-angiogenic factors in the regulation of skeletal muscle angiogenesis. Microcirculation *18*, 316–330.

Pajusola, K., Künnapuu, J., Vuorikoski, S., Soronen, J., André, H., Pereira, T., Korpisalo, P., Ylä-Herttuala, S., Poellinger, L., and Alitalo, K. (2005). Stabilized HIF-1alpha is superior to VEGF for angiogenesis in skeletal muscle via adeno-associated virus gene transfer. FASEB J. *19*, 1365–1367.

Pereira, F.A., Qiu, Y., Zhou, G., Tsai, M.J., and Tsai, S.Y. (1999). The orphan nuclear receptor COUP-TFII is required for angiogenesis and heart development. Genes Dev. *13*, 1037–1049.

Qin, J., Chen, X., Xie, X., Tsai, M.J., and Tsai, S.Y. (2010). COUP-TFII regulates tumor growth and metastasis by modulating tumor angiogenesis. Proc. Natl. Acad. Sci. USA *107*, 3687–3692.

Rowe, G.C., Jang, C., Patten, I.S., and Arany, Z. (2011). PGC-1β regulates angiogenesis in skeletal muscle. Am. J. Physiol. Endocrinol. Metab. *301*, E155–E163.

Salehi, E., Khazaei, M., and Rashidi, B. (2012). Role of fenofibrate in restoring angiogenesis in diabetic and control hind limb ischemic rats. Gen. Physiol. Biophys. *31*, 255–260.

Sarkar, K., Fox-Talbot, K., Steenbergen, C., Bosch-Marcé, M., and Semenza, G.L. (2009). Adenoviral transfer of HIF-1alpha enhances vascular responses to critical limb ischemia in diabetic mice. Proc. Natl. Acad. Sci. USA *106*, 18769–18774.

Sawada, N., Jiang, A., Takizawa, F., Safdar, A., Manika, A., Tesmenitsky, Y., Kang, K.T., Bischoff, J., Kalwa, H., Sartoretto, J.L., et al. (2014). Endothelial PGC-1α mediates vascular dysfunction in diabetes. Cell Metab. *19*, 246–258.

Semenza, G.L. (2009). Regulation of oxygen homeostasis by hypoxia-inducible factor 1. Physiology (Bethesda) *24*, 97–106.

Semenza, G.L. (2012). Hypoxia-inducible factors in physiology and medicine. Cell *148*, 399–408.

Simons, M., and Ware, J.A. (2003). Therapeutic angiogenesis in cardiovascular disease. Nat. Rev. Drug Discov. *2*, 863–871.

Sonoda, J., Mehl, I.R., Chong, L.W., Nofsinger, R.R., and Evans, R.M. (2007). PGC-1beta controls mitochondrial metabolism to modulate circadian activity, adaptive thermogenesis, and hepatic steatosis. Proc. Natl. Acad. Sci. USA *104*, 5223–5228.

van Weel, V., van Tongeren, R.B., van Hinsbergh, V.W., van Bockel, J.H., and Quax, P.H. (2008). Vascular growth in ischemic limbs: a review of mechanisms and possible therapeutic stimulation. Ann. Vasc. Surg. *22*, 582–597.

Varu, V.N., Hogg, M.E., and Kibbe, M.R. (2010). Critical limb ischemia. J. Vasc. Surg. *51*, 230–241.

Wood, S.M., Gleadle, J.M., Pugh, C.W., Hankinson, O., and Ratcliffe, P.J. (1996). The role of the aryl hydrocarbon receptor nuclear translocator (ARNT) in hypoxic induction of gene expression. Studies in ARNT-deficient cells. J. Biol. Chem. *271*, 15117–15123.

Zhang, H., Gao, P., Fukuda, R., Kumar, G., Krishnamachary, B., Zeller, K.I., Dang, C.V., and Semenza, G.L. (2007). HIF-1 inhibits mitochondrial biogenesis and cellular respiration in VHL-deficient renal cell carcinoma by repression of C-MYC activity. Cancer Cell *11*, 407–420.